REMARKS

The Applicants appreciate the Examiner's thorough examination of the subject application. Claims 1, 4, and 5 were pending in the instant application. Claim 6 is new. Support for new claim 6 is found at least at page 12, lines 3-5 of the application as filed. Claim 5 has been amended to depend from claims 1, 4, or 6. Claims 2 and 3 stand cancelled. As such, claims 1, 4, 5, and 6 will be pending upon entry of the within amendment. No new matter is introduced by these amendments.

Applicants make these amendments without prejudice to pursuing the original subject matter of this application in a later filed application claiming benefit of the instant application, including without prejudice to any determination of equivalents of the claimed subject matter.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 1, 4, and 5 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for treating the restenosis or neointimal formation caused by percutaneous transluminal coronary angioplasty (PTCA) or a coronary-artery bypass graft (CABG) with 3-methyl-1-phenyl-2-pyrazolin-5-one, allegedly does not provide reasonable enablement for the therapy of arterial wall injury.

It is suggested that the level of skill in the art, the unpredictability of the art, the amount of guidance and/or working examples, the breadth of the claims, and the amount of experimentation necessary are not described in the specification in such a way to make and/or use the invention.

Applicants disagree and respectfully traverse.

A description is presumed adequate unless sufficient evidence or reasoning is presented to rebut the presumption. See, *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). The courts have provided an objective standard for determining compliance with the written description requirement: "... does the description clearly allow persons of ordinary skill in the art to

recognize that he or she invented what is claimed." *In re Gostelli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (CAFC 1989). Applicants submit that the Action does not provide sufficient evidence to rebut the presumption of Applicants' adequate description. On that basis alone, Applicants submit the rejection is unsupported. Furthermore, Applicants submit that their description does, in fact, provide more than adequate description to support the claimed subject matter.

The instant invention is directed towards the treatment of arterial wall injury which is caused by coronary angioplasty or coronary-artery bypass graft (CABG). Examples 1 and 2 provide for a method of treating an arterial wall injury caused by coronary angioplasty using edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one). The specification clearly indicates that neointimal formation is an arterial wall injury (page 12, lines 3-4 of the specification) and that arterial wall damage by a balloon is a form of coronary angioplasty (page 1, lines 11-20). Examples 1 and 2 also indicate that treatment with edaravone suppresses neointimal formation (page 13, lines 24-27 and page 15, lines 10-20). Therefore, Examples 1 and 2 both provide for the use of 3-methyl-1-phenyl-2-pyrazolin-5-one to treat an arterial wall injury caused by coronary angioplasty.

One of ordinary skill, especially with the combination of background therapeutic knowledge known to one of ordinary skill in the art, as well as the guidance of the specification, would appreciate how to make and use Applicants' claimed subject matter. The Applicants' specification has provided data indicating that arterial wall injuries caused by coronary angioplasty or coronary-artery bypass graft, are treated with 3-methyl-1-phenyl-2-pyrazolin-5-one. Applicants therefore submit that one of ordinary skill in the art would find the specification as filed to be enabling in that the compounds of the invention are clearly delineated, the methods to treat arterial wall injury caused by coronary angioplasty or coronary-artery bypass graft are clearly described, and the methods are well known to those of ordinary skill in the art.

Regarding the alleged lack of enablement regarding certain disorders including hypertension, Applicants submit a copy of Saini, A. K. et al. Pharmacological Research, (2006), 54, 6-10. Saini et al., at page 9, column 2, lines 47-48, observed that edaravone therapy for two weeks results in the normalization of elevated blood pressure.

Withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. § 102(e)

Claims 1, 4, and 5 are rejected as anticipated by Okazaki et al. (US 2004/0242455). It is alleged that Okazaki teaches a composition comprising 5-amidino-N-(2-aminophenethyl)-2-hydroxylbenzesulfonamide derivatives and edaravone for the treatment of restenosis and reocculsion after coronary intervention such as percutaneous transluminal coronary angioplasty or percutaneous transluminal coronary recanalization surgury.

Applicants disagree and restpectfully traverse. Applicants submit that Okazaki is not a valid prior art reference under 35 USC 102(e), because the subject invention was disclosed prior to the effective date of Okazaki.

The instant application was filed in the US on August 18, 2003, and has a USPTO-acknowledged priority date of September 4, 2002.

Regarding 35 USC 102(e)(1), the published Okazaki reference (US 2004/0242455; US 7,022,689) was filed in the US on February 6, 2004. The US filing date of Okazaki is NOT prior to the priority date of the instant application. Regarding 35 USC 102(e)(2), the published Okazaki reference (US 2004/0242455; US 7,022,689) is a US national stage application of PCT/JP02/08093 (WO 03/016269). WO 03/016269 published on February 27, 2003, in Japanese and therefore does not satisfy at least one condition of 35 USC 102(e)(2); specifically, WO 03/016269 did not publish in English.

Section 706.02(f)(1) III of the MPEP provides flowcharts which clearly show that a US National stage application of a PCT international application does not have a 102(e) date if the international application did not published in English. Accordingly, Okazaki is not a valid prior art reference under 35 USC 102(e). The rejection is obviated and Applicants request withdrawal of the rejection.

In view of the above remarks, Applicants believe the pending application is in condition for allowance. Should any of the claims not be found to be allowable, the Examiner is requested to telephone Applicants' undersigned representative at the number below. Applicants thank the Examiner in advance for this courtesy.

The Director is hereby authorized to charge or credit any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105, under Attorney Docket No. 48185-59753, Customer No. 21874.

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Respectfully submitted,

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Edaravone attenuates hydroxyl radical stress and augmented angiotensin II response in diabetic rats

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Abstract

Reactive oxygen species (ROS) potentiate angiotensin II (Ang II) responses in diabetic vasculature. However, superoxide scavengers partially restore this effect, suggesting free radicals other than superoxide could be involved. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is an antioxidant, which primarily scavenges hydroxyl radicals and is approved for use in stroke patients. Hence, to evaluate the role of hydroxyl radical stress in diabetic vascular complications, we studied the effect of edaravone (3 mg kg^{-1}, i.p., b.i.d.) treatment on Ang II responses in thoracic aorta isolated from streptozotocin (60 mg kg^{-1} i.p.) induced 8 weeks diabetic male Sprague–Dawley rats. Ang II (10^{-10} to 10^{-6} M), *tert*-butyl hydro peroxide (tBHP; 10^{-6} to 10^{-3} M) or hydrogen peroxide (H_2O_2; 10^{-6} to 10^{-3} M) induced contractile response was significantly enhanced in aortic strips from diabetic as compared to control rats. Lipid peroxidation was significantly enhanced while the superoxide dismutase (SOD) and catalase activity was significantly lower in aorta of diabetic rats as compared to control rats. Acute (in vitro) exposure of edaravone (10^{-5} M) to aortic strips from diabetic rats in the organ bath restored the augmented Ang II but not tBHP or H_2O_2-induced contractile response. In vivo edaravone (3 mg kg^{-1}, i.p., b.i.d.) treatment for 2 weeks selectively attenuated the augmented Ang II- but not tBHP- or H_2O_2-induced contractile response. The enhanced systolic pressure, lipid peroxidation and the reduced SOD and catalase activity were restored to control values following 2 weeks edaravone treatment. From our results we infer that hydroxyl radical stress augments Ang II response in diabetic rat thoracic aorta and edaravone could be an ideal antioxidant adjuvant in the therapy of diabetic vascular complications.

Keywords: Angiotensin; Diabetes; Edaravone; Hydroxyl radical; Streptozotocin

1. Introduction

Vascular complications associated with the diabetes are major cause for the increased morbidity and mortality in diabetic patients [1–6]. Angiotensin II (Ang II) induced AT_1 receptor mediated altered vascular structural and functional physiology [1,2,7], in addition to the direct effects of hyperglycemia [4,8], are evident to be a major factor in the development of the vascular complications. Although Ang II as well as hyperglycemia induced superoxide formation is a key event in the vascular pathophysiology [6], several reports including recent data from our lab, indicate that superoxide scavengers partly revert the enhanced Ang II response in diabetic animals [1,7]. Such partial effects suggest that radicals other than superoxide may be involved in the vascular pathophysiology.

Ang II signaling occurs mainly via AT_1 receptors [9] with increasing evidence suggesting that NADPH oxidase-dependent generation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2) and the reactive hydroxyl radical may be early events [6,10–13]. The superoxide anion generated is converted by superoxide dismutase (SOD) to H_2O_2, while hydroxyl radical is produced by the fenton reaction [14–16]. ROS mediate proliferative/hypertrophic responses to Ang II,

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both in vivo and in vitro [11,12,14,17]. Although their role in Ang II-dependent vascular contraction is not conclusive, the involvement of superoxide anion, H_2O_2 and hydroxyl radicals are proposed under pathophysiology [10,18]. Previous reports from our lab indicate that superoxide anion partly contributes to the enhanced vascular contractile response to Ang II in experimental models of diabetes [1,7] and hypertension [19]. Tempol, a membrane-permeable, water-soluble SOD mimetic (superoxide anion scavenger) partially reverses the augmented Ang II-induced vasoconstriction in thoracic aorta of STZ-induced diabetic rats [1]. This prompted us to speculate the role for other radicals (probably hydroxyl radical) in the enhanced vascular response to Ang II, which we aimed to address in the current study. Edaravone (a hydroxyl radical scavenger) is an approved drug for the treatment of stroke [20,21]. We have used this drug as a tool to study the role of hydroxyl radical in the enhanced response to Ang II in diabetic vasculature and report its beneficial effects, suggesting the role of hydroxyl radical stress in diabetic vasculopathy and also being an approved drug the possibility of extending the study in diabetic patients.

2. Materials and methods

2.1. Animals, induction of diabetes and edaravone treatment schedule

Male Sprague–Dawley rats (160–180 g) were procured from Central Animal Facility, NIPER. Experimental protocols were approved by the Institutional Animal Ethics Committee of NIPER. Plasma glucose levels were measured in all animals before administration of streptozotocin (STZ). Animals showing plasma glucose levels in the range of 3.9–5.5 mM measured by GOD–POD plasma glucose diagnostic kit (Accuron, India), were included in study. Rats were made diabetic using STZ as described previously [2,7]. Rats with a blood glucose level >20 mM were selected for the study. Eight weeks post-induction of diabetes the rats were sacrificed under ether anesthesia and the thoracic aorta was isolated for organ bath studies and estimation of biochemical parameters. A randomly selected group of 6 weeks diabetic rats were treated with edaravone (3 mg kg^{-1}, i.p., b.i.d.) for 2 weeks, while the control rats received vehicle (isotonic saline, i.p., b.i.d). Edaravone dose was selected based on preliminary studies using 1, 3 and 10 mg kg^{-1}, i.p., b.i.d (data not shown). To assess the possible cardiovascular effects of edaravone, blood pressure and heart rate were measured non-invasively using IITC tail cuff probe (USA) as described before [1].

2.2. Chemicals

Edaravone (MCI-186; 3-methyl-1-phenyl-2-pyrazolin-5-one) was purchased from Calbiochem, Germany; GOD/POD glucose kit from Accurex, India; streptozotocin, tBHP from Sigma Chemical Co., St. Louis, USA; H_2O_2 from Merck, India; angiotensin II from Bachem, Basel, Switzerland. All other chemicals were of reagent grade, purchased locally.

2.3. Biochemical analysis

2.3.1. Assay for SOD activity

Isolated thoracic aorta was cleaned of surrounding fat and homogenized in 50 mM PBS buffer pH 7.0 using polytron homogenizer. Homogenate was then centrifuged at 4 °C, 15,000 rpm for 10 min. Supernatant was used for the estimation of SOD activity by hematoxylin auto-oxidation method as described [1].

2.3.2. Assay for catalase activity

Catalase activity was measured according to Grover et al. [22]. Thoracic aorta was homogenized (20 mg of tissue/ml of PBS, pH 7.1) and centrifuged at 4 °C (15,000 rpm for 10 min). The supernatant obtained was used for the assay. The degradation pattern of exogenously added H_2O_2 by catalase enzyme present in 200 μl of tissue supernatant was monitored at 240 nm in spectrophotometer at 15 s interval for 5 min and its activity calculated. Catalase activity is expressed as U/mg of protein. Protein was estimated by Lowry's method.

2.3.3. Lipid peroxidation assay

The concentration of MDA (thiobarbituric acid reactive substance (TBARS)) was assayed using the method described by Beltowski et al. [23]. 0.5 ml of plasma or 1 ml of tissue supernatant of thoracic aorta was mixed with 1 ml of 10% trichloroacetic acid and allowed to stand for 30 min at 37 °C. Then 1 ml of 0.67% (w/v) thiobarbituric acid and 20 μl of 20% BHT and the sample were heated at 95 °C for 30 min in boiling water bath. After cooling to room temperature, 2 ml of n-butanol was added and vortex immediately and centrifuged for 5 min at 5000 rpm. The organic layer was removed and its absorbance was measured at 532 nm. The concentration of MDA is expressed as nM of MDA/mg of tissue (aorta).

2.4. Vascular reactivity to Ang II, H_2O_2 and tBHP

Eight weeks post-STZ administration, the rats were sacrificed and thoracic aorta was isolated from the heart to the diaphragm and cleaned of surrounding fat and connective tissues. Care was taken not to stretch the vessel. Helical strips of aorta of 3 mm in width and 20 mm in length was cut with sharp iris scissors and placed in 10 ml organ bath containing Krebs–Henseleit buffer (NaCl 118 mM; KCl 4.7 mM, KH_2PO_4 1.2 mM, Mg $SO_4 \cdot 7H_2O$ 1.2 mM, $CaCl_2 \cdot 2H_2O$ 2.5 mM, $NaHCO_3$ 25 mM and glucose 5.5 mM) of pH 7.4 and osmolality (280–308 mOsmol). The solution was continuously aerated with 5% carbogen at 37 °C. A resting tension of 2 g was applied to the strips and allowed to equilibrate for 2 h. After 2 h of equilibration, two wake up responses of KCl (80 mM) were recorded following which concentration response curves (CRC) of Ang II (10^{-10} to 10^{-6} M), H_2O_2 (10^{-6} to 10^{-3} M) and tBHP (10^{-6} to 10^{-2} M) were recorded in absence or presence of edaravone (10^{-5} M). Changes in the isotonic contraction were recorded as described [1,2,8]. The maximum vasoconstrictor response to the respective agonists in control tissue was considered as 100%.

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical comparisons were performed with one-way ANOVA followed by post hoc (Bonferroni's) test. A p value < 0.05 was considered significant. All statistical tests were performed using the Prism software package (version 4, GraphPad, San Diego, CA, USA).

3. Results

3.1. Antihypertensive and antioxidant effects of edaravone in diabetic rats

STZ-administered rats developed symptoms of type 1 diabetes as previously described [1]. Due to prolonged hyperglycemia (8 weeks), oxidative stress was observed in these animals, which was supported by decrease in catalase and SOD activity and elevation in lipid peroxidation. Oxidative stress generated by hyperglycemia leads to vascular complications like hypertension, which correlated well with increase in systolic blood pressure (Table 1). Two-week edaravone treatment significantly restored systolic blood pressure and lipid peroxidation to normal and enhanced the catalase and SOD activity in diabetic rats (Table 1).

3.2. Edaravone selectively inhibits augmented response of angiotensin II but not H_2O_2 or tBHP

Ang II-, H_2O_2- and tBHP-induced contraction in endothelium intact aortic spiral preparations were significantly enhanced in thoracic aorta from diabetic rats as compared to age matched control rats as evident by supersensitivity (increase in pD_2 value) and increase in maximal response (E_{max}) (Figs. 1–3). Preincubation of blood vessel with edaravone (10^{-5} M) for 15–20 min significantly restored the enhanced response to Ang II but not to that of H_2O_2 or tBHP in thoracic aorta from diabetic rats, however, it did not influence the response to any of these spasmogens in thoracic aorta from control rats. Similar trend in response to these spasmogens were observed in thoracic aorta isolated from diabetic rats treated with edaravone for 2 weeks

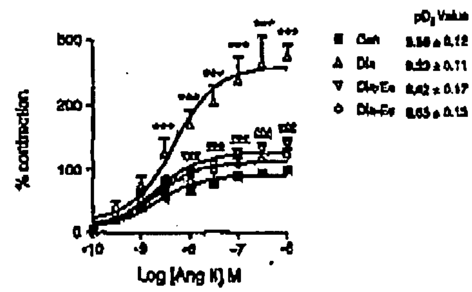

Fig. 1. Cumulative concentration response curves and pD_2 values of Ang II in endothelium intact aortic spiral preparations obtained from age matched control (Con), 8 weeks diabetic (Dia) rats, in vitro edaravone (Dia + Ex; 10^{-5} M, for 15–20 min) treated vessels from diabetic rats and 2 weeks edaravone (Dia + Ec; 3 mg kg^{-1}, i.p., b.i.d.) treated diabetic rats. Each value is represented as mean ± S.E.M., $n = 6$, $^{***}p < 0.001$ vs. control. $^{£££}p < 0.001$ vs. diabetic.

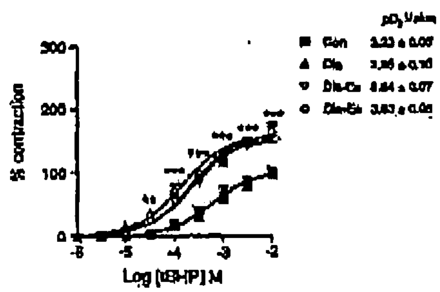

Fig. 2. Cumulative concentration response curves and pD_2 values of tBHP in endothelium intact aortic spiral preparations obtained from age matched control (Con), 8 weeks diabetic (Dia) rats, in vitro edaravone (Dia + Ex; 10^{-5} M, for 15–20 min) treated vessels from diabetic rats and 2 weeks edaravone (Dia + Ec; 3 mg kg^{-1}, i.p., b.i.d.) treated diabetic rats. Each value is represented as mean ± S.E.M., $n = 6$. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

(Figs. 1–3). Selectivity of edaravone to inhibit the augmented Ang II response both in vitro as well as in vivo suggests the involvement of hydroxyl radical stress in augmented responses of Ang II in diabetic animals.

Table 1
Effect of edaravone on body weight, blood pressure and biochemical parameters

	Control	Diabetic	Diabetic + edaravone
Body weight (g)	348 ± 5.7	183 ± 5.8***	202 ± 5.2£
Plasma glucose (mg/dl)	91 ± 2.7	446 ± 7.2***	463 ± 13.8
Systolic Blood pressure (mmHg)	121 ± 2	158 ± 4***	128 ± 3£££
Heart rate (beats/min)	377 ± 13	388 ± 34	391 ± 28
SOD activity (U/mg protein)	28.9 ± 5.4	0.68 ± 0.3***	31.8 ± 4£££
Catalase activity (U/mg protein)	2.6 ± 0.02	0.37 ± 0.02***	2.14 ± 0.04£££
Lipid peroxidation (μM MDA/mg protein)	1.77 ± 0.03	4.8 ± 0.6***	2.1 ± 0.2£££

Each value is represented as mean ± S.E.M., $n = 8$–10.
£ $p < 0.05$ vs. diabetic group.
£££ $p < 0.001$ vs. diabetic group.
*** $p < 0.001$ vs. control.

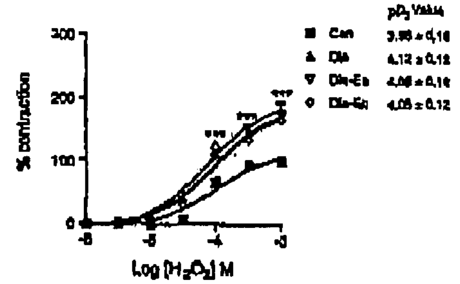

Fig. 3. Cumulative concentration response curves and pD_2 values of H_2O_2 in endothelium intact aortic spiral preparations obtained from age matched control (Con), 8 weeks diabetic (Dia) rats, in vitro edaravone (Dia + Ea; 10^{-3} M, for 15–20 min) treated vessels from diabetic rats, and 2 weeks edaravone (Dia + Ec; 3 mg kg^{-1}, i.p., b.i.d.) treated diabetic rats. Each value is represented as mean ± S.E.M., $n = 6$. $^{***}p < 0.001$ vs. respective control group.

4. Discussion

The pathophysiological relevance of exogenous antioxidant therapy is envisaged by the discovery of reactive oxygen species generators (NADPH oxidases, xanthine oxidase, uncoupled nitric oxide synthases, lipoxygenases and mitochondrial electron transport complex) in vasculature [16,17]. Ang II is a crucial hypertrophic/hyperplastic factor in vascular wall, contributing to several pathophysiological conditions [12,24,25]. These actions of Ang II are related to a peptide-dependent increase in ROS synthesis [16].

Increased generation of superoxide anion and other ROS and decreased plasma or tissue concentrations of superoxide dismutase, catalase, glutathione and ascorbic acid are reported in both clinical and experimental diabetes [26,27]. Amongst the ROS, superoxide anion, hydoxyl radical and H_2O_2 [28,29] are implicated in the impaired relaxation responses to acetylcholine (a marker for endothelial function) [6,11]. Thus, conclusively establishing the role of ROS in the endothelial dysfunction. Similarly, we observed the involvement of superoxides in the enhanced contractile response to Ang II in aortic rings obtained from SHR [19,30] and diabetic rats [1,7]. While interventions/treatments with superoxide scavengers restores endothelial function, Ang II-induced enhanced contraction is partially improved, suggesting the role of other ROS. Recent reports show the involvement of H_2O_2 in mediating the hypertrophic and contractile responses to Ang II [15,31–33]. However, studies exploring the involvement of H_2O_2 in pathological condition like diabetes are bidirectional. While some believe lesser involvement of molecular H_2O_2 in pathogenesis of the diabetes [34], others predict its predominant involvement owing to its membrane permeability [28,32,33,35]. However, the fact that H_2O_2 can react with transition metal Fe^{2+} to produce highly reactive hydroxyl radicals (Fenton reaction) and hypochlorous acid (generated by the myeloperoxidase) react with superoxides, further contribute to hydroxyl radical stress [17,18] is largely overlooked in the context of diabetic pathophysiology. Hydroxyl radical is the highly reactive and deleterious ROS and it has been shown that hydroxyl radicals can lead to endothelial dysfunction [18], hence its involvement in mediating augmented Ang II response is more likely than those of other ROS. Several synthetic compounds, which efficiently scavenge the hydroxyl radical, are used in stroke therapy. Edaravone is one such drug [20,36], which is approved for the treatment of stroke [21] and reported to protect hydroxyl radical-induced ischemic reperfusion injuries [37–40]. Hence, we studied the involvement of hydroxyl radical in the enhanced contractile responses to Ang II in diabetes using edaravone. In vitro exposure as well 2 weeks in vivo treatment with edaravone restored the augmented Ang II responses, suggesting hydroxyl radical stress augments the Ang II responses in diabetes, which may be major factor for cardiovascular dysfunction in diabetes. To best of our knowledge this is the first study showing that hydroxyl radical mediate the augmented Ang II vascular response in diabetes. We have also checked the selectivity of the edaravone to Ang II response, by studying its effects on responses to H_2O_2 and tBHP. We did observe supersensitivity (increase in pD_2 value) as well as greater contraction (increase in E_{max}) of aortic spiral preparation to tBHP (Fig. 2) and H_2O_2 (Fig. 3) in diabetic rats as compared to age matched control rats, which was not influenced by in vitro or in vivo edaravone treatment. This differential effect of edaravone on Ang II, H_2O_2 and tBHP, suggests that supersensitivity of the contractile elements to ROS is also a feature in diabetic vasculature, hence identifying the specific ROS, their molecular source and evaluating their signaling cascade is crucial in understanding the disease process. A step towards this our study shows that hydroxyl radicals are the primary ROS involved in Ang II-induced supersensitivity in diabetic vasculature. Here it is important to note that increase in catalase activity with edaravone treatment for 2 weeks, did not restore the augmented H_2O_2 responses in diabetic aorta. This may be due to the change in sensitivity of the contractile elements to H_2O_2 in diabetic condition, which may not be influenced by treatment with antioxidants or specific radical scavengers or increase in antioxidant enzyme defense.

Consistent to our observation of hydroxyl radical stress in enhanced response to Ang II in diabetes, edaravone treatment for 2 weeks significantly restored the catalase and SOD activity, lipid peroxidation and systolic blood pressure to normal in STZ-induced diabetic rats, which suggest the role of hydroxyl radical stress in diabetic vascular complications. The mechanisms behind the changes observed in blood pressure could be hypothesized as hydroxyl radicals mediate to be initiation factors/early events. Our observation that edaravone therapy for 2 weeks could effectively normalize the elevated blood pressure does support this hypothesis but needs additional and well-controlled and time-dependent studies to arrive at a conclusion.

In conclusion, the present experiments point to hydroxyl radical as a critical mediator of the augmented Ang II responses in diabetic rat thoracic aorta and edaravone selectively attenuates augmented Ang II responses in diabetic rat thoracic aorta, which is selectively attenuated by edaravone. Hence, edaravone could be a promising adjuvant antioxidant therapy for vasculopathy associated with diabetes.

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